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The Kinetics of the Conductance Increase Produced by γ-Aminobutyric Acid at the Membrane of Locust Muscle Fibers

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SUMMARY

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The membrane response to γ-aminobutyric acid (GABA) of locust muscle fibers bathed in "propionate saline" at pH 6.5 is generally biphasic. The slow phase is exponential, and its rate constant (5.5×10^{-3} to 1.0×10^{-2} sec^{-1}) is independent of GABA concentration and relatively insensitive to temperature change (from 26° to 15°). With increasing GABA concentration and temperature, a faster process intervenes. A form of "sensitization" occurs in which the participation of the fast component of the kinetics increases on repeated application of the same GABA concentration. Equilibrium curves of GABA action were obtained at different temperatures, and a van't Hoff plot was constructed. The standard enthalpy change, $\Delta H°$, for the over-all interaction between GABA and receptor is −59.3 kcal/mole.

INTRODUCTION

In experiments designed to measure equilibrium responses to the action of γ-aminobutyric acid (1), the membrane of locust muscle fibers was found to respond very slowly to the presence of γ-aminobutyric acid added to the bath. Several minutes were required before the membrane conductance of a superficial fiber reached a new steady value. The inhibitory neurotransmitter action of GABA[2] in arthropods is known to be slower than the excitatory action of glutamate (2). Nonetheless, when GABA is added to the solution bathing crayfish muscle fibers, 90% of the equilib-

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[1] On sabbatical leave as Visiting Professor at the Hebrew University.

[2] The abbreviation used is: GABA, γ-aminobutyric acid.

rium response is reached within 20–50 sec (3). Under similar experimental conditions the rate of action of acetylcholine and acetylcholine-like drugs at the motor end plate of frog muscle is as fast or faster (4, 5). The very slow responses measured in this work were therefore quite unexpected.

We describe below the results of experiments in which the kinetics of GABA action on the membrane of locust muscle fibers was measured at different temperatures. The observed variation of an apparent equilibrium constant with temperature has permitted the calculation of thermodynamic parameters for the interaction. The details of the kinetics are sufficiently complex that only a descriptive treatment is attempted at this stage.

A preliminary report has been given of some of these results (6).

METHODS

The preparation used in this study was the flexor tibialis muscle of the metathoracic (jumping) leg of *Locusta migratoria*. A description of the preparation and of experimental procedures for the measurement of membrane conductance has been given in the preceding paper (1). Here we deal with aspects relevant to the measurement of kinetics.

In the present experiments a modified superfusion chamber was used with a reduced bath volume of 0.3 ml. Solution flowed continuously through this chamber, superfusing the muscle fibers at a rate of 20 ml/min. The tap for exchanging solutions was located close to the chamber. The rate rate of exchange of solutions in the chamber on operating the tap was checked by observing the clearance of saline solution by distilled water. The resistance between two platinum wire coils clamped in the chamber was measured. Exchange was 95% complete in 3.5 sec and 99% complete in 7 sec. It was also possible to ascertain that the solution in contact with the muscle did not remain stationary while flow took place around the preparation. When, at the end of an experiment, a little concentrated saline solution was injected into the flow tube leading to the chamber, lines of flow of the solution became momentarily visible because of the difference between the refractive indices of dilute and concentrated salt solutions.

A superficial muscle fiber was impaled with two micropipettes approximately one fiber diameter apart (1). Linear ramps of inward current were passed across the membrane through one micropipette, and the electrotonic potential change was detected by the other. The kinetics of the membrane response to GABA was recorded on an *xy* plotter, current to the *y* input and potential to the *x* input. The current ramps were of 1.6-sec duration and were usually applied at 10-sec intervals. Input conductance (hereafter referred to as conductance) was read from the slope of the line written out by the plotter. The pen was shifted manually along the *y* axis between current ramps (Fig. 1).

The bathing solution used in these experiments was "propionate saline" (1) containing C_2H_5COONa (112 mM), NaCl (28 mM), KCl (10 mM), $CaCl_2$ (2 mM), and $MgCl_2$ (2 or 4 mM). The solution was adjusted to pH 6.5 by addition of propionic acid. A calibrated thermistor probe (type 421, Yellow Springs Instrument Company) was used to monitor the temperature of the solution in the chamber.

RESULTS

Resting conductance. It has been shown previously for arthropod muscle that the properties of the resting membrane and its response to GABA are very sensitive to temperature (7, 8). In the present work a reduction in temperature from room temperature (20–26°) to 15° caused a substantial decrease in resting membrane conductance and a depolarization of several millivolts. The extent of the conductance change (Fig. 2) agrees rather precisely with the value of 5%/degree reported for crab muscle (7).

Equilibrium responses. At reduced temperature measurable increases in membrane conductance could be elicited with smaller concentrations of GABA. In Fig. 3 log dose-response curves measured in different preparations at 15° and 24.5° are seen to be approximately parallel. The responses in Fig. 3 are expressed as percentages of maxima, which were not measured experimentally but were extrapolated by the method

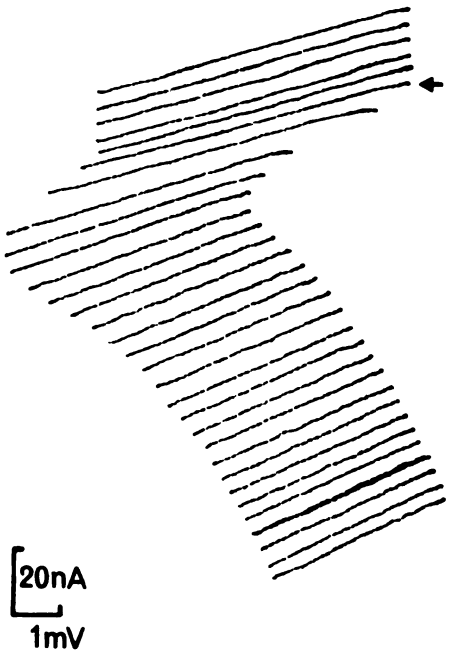

FIG. 1. *xy plotter recording of rate of increase of membrane conductance of an impaled fiber on application of GABA*

The zero point of each line is on the right, and the pen of the *xy* plotter lifts at the peak of the current ramp. The pen was shifted manually down the *y* axis (current axis) between current ramps, which were applied at intervals of 10 sec. From the arrow downwards the preparation is superfused with 0.24 mM GABA. The response consists of a transient hyperpolarization of the membrane, recorded as a displacement of the zero points to the left, and a sustained conductance increase, recorded as a change in the slope of the lines. The lines also become shorter because the peak current produces less hyperpolarization when conductance increases. In this response the slopes of the first two lines after the arrow would be considered indeterminate because of distortion by the fast rate of GABA-induced hyperpolarization.

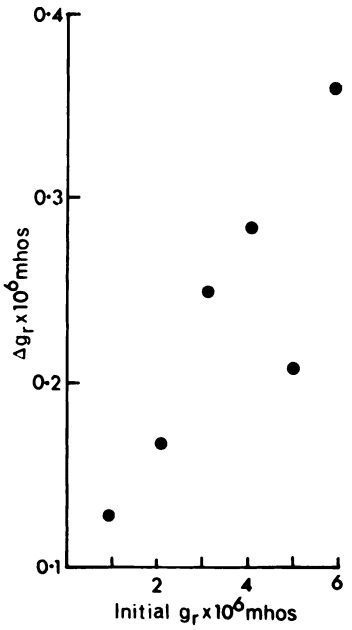

FIG. 2. *Effect of temperature on resting membrane conductance*

The relationship between g_r, the initial resting conductance of the muscle fiber membrane at room temperature (23–26°), and Δg_r, the conductance decrease per degree produced by cooling the bathing solution to 15–19°, is shown. The points represent single observations of the effect of cooling on g_r made in each of six preparations. The slope of the regression line through the points is 0.048 ± 0.003 (standard error of estimate).

described in the preceding paper (1). This method depends upon the fact that the equilibrium curve of GABA action is approximated by the equation for an *n*th-order reaction:

$$y = \frac{[A]^n}{[A]^n + K} \qquad (1)$$

where $[A]$ is GABA concentration, K is an apparent equilibrium dissociation constant, and y is directly proportional to g', the equilibrium conductance increase. The value of n is not defined here as an integral number of binding sites, but is found empirically by minimizing the sum of squares of deviations from linearity in the double-reciprocal plot of $1/g'$ vs. $1/[A]^n$. The justifications for the important assumptions made in this approach have been discussed elsewhere (1). The approximate fit given by Eq. 1 to the data makes it possible to characterize each dose-response curve by only three quantities: (a) the maximum response, g_{max}; (b) the concentration at half-maximum response, $[A]_{0.5}$; and (c) the Hill slope, $d \log[y/(1 - y)]/(d \log[A])$, which in this case is equal to n.

In three determinations at 15° the Hill slope was 2.6, 3.3, and 3.0. These slopes are

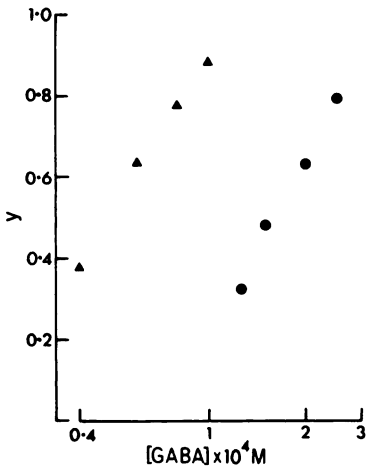

FIG. 3. *Log dose-response curves at 15° and 24.5°*
Responses are expressed as fractions of an extrapolated maximum conductance increase. The curves were measured in different preparations at 15° (▲) and 24.5° (●).

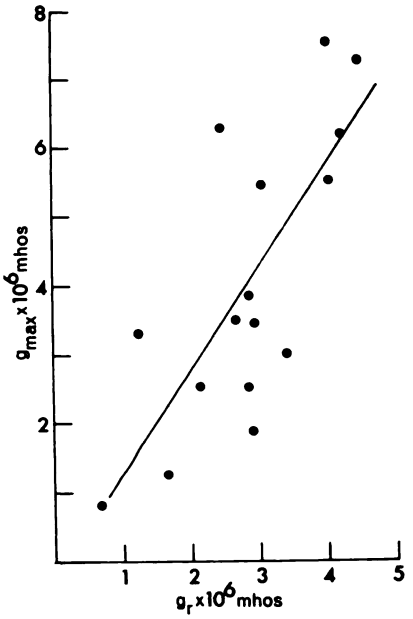

FIG. 4. *Relationship between resting membrane conductance and maximum response to GABA*
The maximum conductance increases, g_{max}, are extrapolated values (see the text) from experiments at temperatures between 15° and 26°. The linear regression of g_{max} on g_r is shown [slope, 1.49 ± 0.35 (standard error of estimate)], since g_{max} is considered the dependent variable. Because of the scatter of the points, the regression of g_r on g_{max} gives a steeper relationship.

therefore not different[3] from the mean value of 2.80 ± 0.24 (standard error) determined in propionate saline at room temperature (1). Figure 4 shows that the extrapolated values of g_{max} increase with resting conductance, g_r. An inverse relationship was reported for crab muscle (7). In the present work, using propionate saline at pH 6.5, there is a tendency for the ratio $g_{max}:g_r$ to remain roughly constant in the region of 1.5. On reduction of temperature g_{max} thus becomes smaller in direct proportion to g_r. It seems clear, therefore, that the increase in sensitivity to GABA action at reduced temperature is caused by an increase in affinity, as indicated by the parallel shift of the log dose-response curve shown in Fig. 3.

[3] The Mg^{++} content of the saline was 4 mM in some experiments in which kinetics was measured, whereas in the majority of experiments the content was 2 mM. Hill slopes unequivocally greater than 2.8 were measured for the first time under these conditions of increased Mg^{++} concentration. The usual lower value slope, however, was measured at reduced temperature. For instance, the Hill slope for the experiment shown in Fig. 7 (23°) was 4.1, while that for the experiment in Fig. 10 (15°) was 2.6.

Van't Hoff plot. The empirical use of Eq. 1 to describe the interaction of GABA with receptor provides us with an apparent overall affinity constant, K^{-1}, for the interaction. Since the best estimate of n is 2.8 (1), we may write $K = [A]_{0.5}^{2.8}$.

Figure 5 shows a van't Hoff plot of $-\log K$ against the reciprocal of absolute temperature, T, for 21 determinations of $[A]_{0.5}$ at various temperatures between 26° and 15°. Each value of $[A]_{0.5}$ was taken from a Hill plot ($\log [y/(1-y)]$ vs. $\log [A]$) constructed on the basis of an extrapolated maximum.

The order of application of GABA concentrations can influence the equilibrium values of conductance increase (1). Different symbols were used for the points in Fig. 5 according to the following categories: (a) ascending order of concentrations without intermediate washout, (b) descending order of concentrations without intermediate

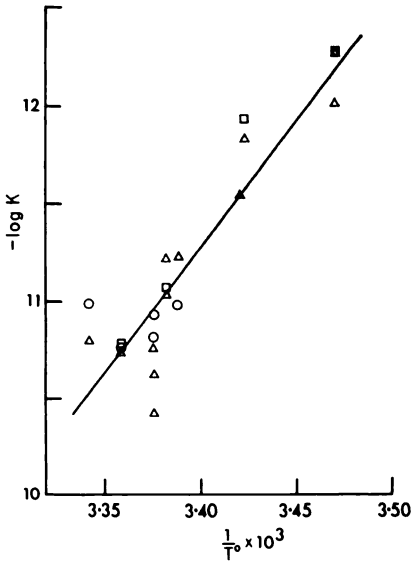

FIG. 5. *van't Hoff plot for interaction between GABA and receptor*

The negative logarithm of the estimated dissociation constant, K, is plotted against the reciprocal of the absolute temperature. The experimental equilibrium curves used to estimate K were obtained in different ways: △, ascending order of concentrations without intermediate washout; ○, descending order of concentrations without intermediate washout; □, intermediate washout and recovery between concentrations. The three sets of data appear to be included in a single population. The slope of the regression line is 12.95 ($\pm$1.36) $\times$ 10^3.

washout, and (c) intermediate washout and recovery between concentrations. However, inspection of Fig. 5 suggests that all the points in fact belong to the same population.

The standard enthalpy change, $\Delta H°$, for the over-all interaction of GABA with receptor was calculated from the slope of the regression line in Fig. 5 by use of the following expression:

$$\Delta H° = -\text{slope} \times 2.303 \times R$$
$$= -59.3\ (\pm 6.2)\ \text{kcal/mole}$$

where R is the gas constant.

The other thermodynamic parameters of the interaction were calculated as follows: Gibbs free energy change, $\Delta F° = RT \ln K$ (note that the affinity constant is K^{-1}); entropy change, $\Delta S° = (\Delta H° - \Delta F°)/T$. The calculated values are given in Table 1.

These values apply to an unknown sequence of equilibria leading to the formation of activated receptor. Insofar as they are similar to the thermodynamic parameters for the interaction of, for example, adenosine 5-monophosphate with fructose 1,6-diphosphatase (9), or of oxygen with hemoglobin (10), they are consistent with the idea that the GABA receptor is a multi-subunit protein (1).

Kinetics. The rate of change of membrane conductance in response to the application of GABA is hard to discern from the original xy plotter recordings (Fig. 1). Figure 6 illustrates the form of the responses when membrane conductance is plotted against

TABLE 1

Thermodynamic parameters for interaction between GABA and receptor ($\Delta H° = -59.3$ *kcal/mole*)

The data are averaged from three to five experiments.

Temperature	$[A]_{0.5}$	$\Delta F°$	$\Delta S°$
°C	$M \times 10^4$	*kcal/mole*	*e.u./mole*
23	1.52	−14.5	−151
19	0.63	−15.7	−149
15	0.45	−16.1	−150

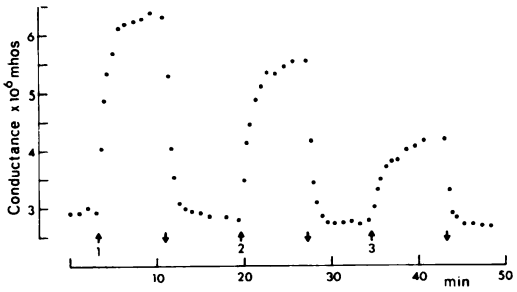

FIG. 6. *Form of responses to GABA*

The slope of each line in an original recording of the kind shown in Fig. 1 is converted into a point in this plot of membrane conductance against time. The upward arrows indicate applications of GABA (*1*) 100 μM, (*2*) 70 μM, and (*3*) 50 μM, and the downward arrows indicate washout of the GABA. The first three points following each arrow are at intervals of 20 sec. The experiment was performed at 19°, and these responses were recorded after several previous applications of GABA to the preparation. The largest of the three responses is approximately 80% of maximum.

time. In view of the experimental conditions of recording from a superficial fiber in contact with rapidly moving solution, these responses must be considered remarkably slow.

The use of a semilogarithmic plot (Fig. 7) shows the kinetics frequently to be biphasic. In Fig. 7 are plotted onsets (A) and offsets (B) of the action of four submaximal concentrations of GABA. The onsets resolve into fast and slow exponential phases, and can therefore be described by an equation of the form

$$1 - \frac{g}{g'} = a_0 e^{-k_a t} + b_0 e^{-k_b t} \qquad (2)$$

where g is the instantaneous conductance change at time t; g' is the equilibrium conductance change at infinite time; a_0 and b_0 are the initial fractions of the fast and slow components, respectively (such that $a_0 + b_0 = 1$); and k_a and k_b are the rate constants of the fast and slow processes.

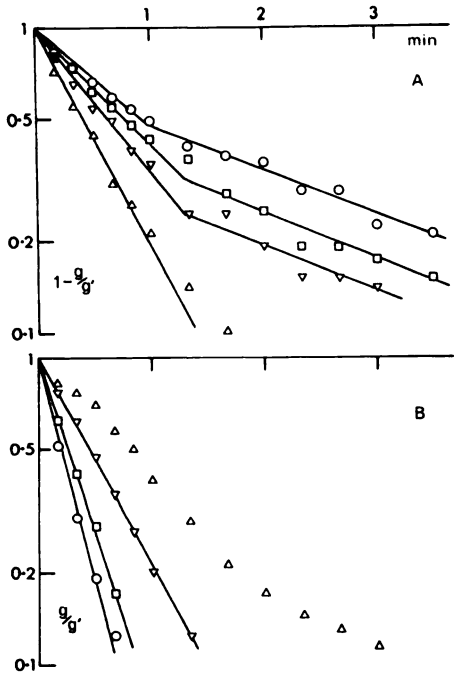

FIG. 7. *Kinetics of onset (A) and offset (B) of GABA action at room temperature (23°)*

The instantaneous conductance increase, g, was measured at multiples of 10 sec after application of GABA (see Fig. 1) until equilibrium conductance increase, g', was reached. On washout, the kinetics of offset of GABA action was also followed. Each GABA application was of 7-min duration, and each washout was of 6-min duration. The logarithmic axis of the onset kinetics is plotted as $(1 - g/g')$, and that of the offset kinetics as g/g', to facilitate comparison. The GABA concentrations applied were: ○, 100 μM; □, 150 μM; ▽, 190 μM; △, 240 μM ($[A]_{0.5}$ = 140 μM). The half-times of the fast and slow exponential components of the onsets are given in Table 2. All responses were recorded from the same fiber.

The rate constants and initial fractions of the fast and slow components were determined graphically (11). Table 2 shows that the half-times of both components are independent of GABA concentration. The concentration dependence of the onsets is thus entirely accounted for by the increase in the proportion of fast component as concentration increases. An analysis of this kind cannot be applied when, as often happens, the fast phase does not appear to be exponential (Fig. 10).

"*Sensitization.*" The first few applications of GABA to a preparation elicit responses which become progressively faster. The effect is more marked at higher GABA concentration. This initial tendency towards increased rate of action of GABA has been termed "sensitization" (12). The effect is entirely a kinetic one, since the conductance increase at equilibrium shows no consistent trend on repeated application of the same concentration of GABA. Figure 8 suggests that sensitization involves increasing participation

TABLE 2

Half-times of fast and slow exponential components of response to GABA at 23°

Equation 2 was used to analyze the kinetics of the onsets of GABA action in the experiment shown in Fig. 7.

GABA	$y = g'/g_{max}$	a_0	Half-time of k_a process	Half-time of k_b process
$M \times 10^4$			*sec*	*sec*
1.0	0.19	0.34	—[a]	126
1.5	0.51	0.49	22	117
1.9	0.78	0.62	22	125
2.4	0.92		23	

[a] Deviation from linearity prevented determination of a reliable value (see Fig. 7).

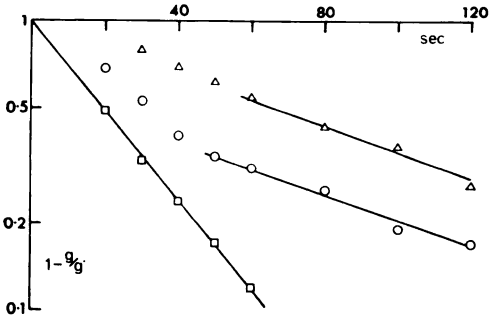

FIG. 8. *Kinetics of initial responses to repeated application of the same GABA concentration ("sensitization")*

The first (△), second (○), and third (□) responses to 240 μM GABA in a previously untreated preparation are shown superimposed in a semilogarithmic plot (see legend to Fig. 7). The points close to the origin were indeterminate because of rapid hyperpolarization of the kind shown in Fig. 1. The first application of GABA was made 3 hr after dissection of the preparation. GABA was applied for about 10 min and washed out for a similar period. The equilibrium responses, g', were: △, 2.6×10^{-6} mho; ○, 2.9×10^{-6} mho; □, 2.6×10^{-6} mho. The experiment was performed at room temperature (24.5°). The rate constants of the fast and slow exponential processes, indicated by lines, are 3.6×10^{-2} sec^{-1} and 1.0×10^{-2} sec^{-1}, respectively.

of a fast kinetic component and decreasing participation of a slow component. The first application of GABA in the experiment shown in Fig. 8 was made after equilibrating the muscle in propionate saline for 3 hr. Each of the three responses was accompanied by transient hyperpolarization (2–3.5 mV) followed by depolarization, with subsequent repolarization on washout of the GABA.

No attempt was made to determine whether the kinetics would become slower again after a long period of recovery in the absence of applied GABA. The responses shown in Fig. 7 were recorded after sensitization of the preparation, and the smallest concentration was reapplied at the end of the series to establish reproducibility of the kinetics.

Temperature dependence of kinetics. Application of the same concentration of GABA at different temperatures yielded the surprising result that the rate of GABA action increased with decreasing temperature. In fact, this observation is misleading, since the ratio $[A]:[A]_{0.5}$ increases as the temperature is lowered, and a concentration which is submaximal at room temperature may be supramaximal at 15°. To obtain good estimates of $[A]_{0.5}$ at different temperatures in the same experiment was difficult. We have therefore taken the less satisfactory course of making comparisons between experiments at different temperatures. Figure 9 shows onset kinetics at 15°, 19°, and 24.5° with the ratio $[A]:[A]_{0.5}$ approximately equal to 1. This figure illustrates our general finding that the participation of the fast component decreases at reduced temperature.

The rate constant of the slow component appears from Fig. 9 to decrease with decreasing temperature (the values are 7.3×10^{-3} sec^{-1} at 24.5°, 6.3×10^{-3} sec^{-1} at 19°, and 5.5×10^{-3} sec^{-1} at 15°). However, Fig. 10 shows another experiment at 15°, in which the rate constant of the slow component is 7.7×10^{-3} sec^{-1}, and a value of 5.5×10^{-3} sec^{-1} is found in the experiment at 23° shown in Fig. 7. Thus the effect of temperature on the rate constant of the slow component is obscured by experimental variation, and the indication is that this process has a rather small Q_{10}.

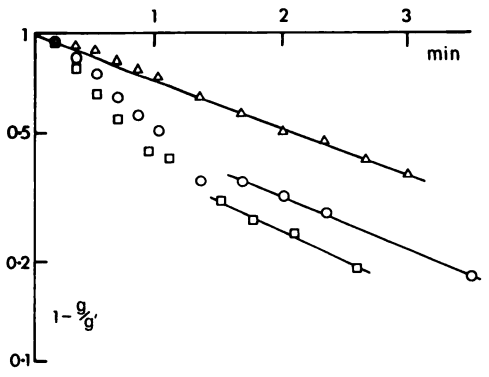

FIG. 9. *Effect of temperature on kinetics of onset of GABA action*

The method of plotting is explained in the legend to Fig. 7. Each onset is taken from a different experiment: △, 58 μM ($[A]_{0.5}$ = 41 μM) at 15°; ○, 48 μM ($[A]_{0.5}$ = 55 μM) at 19°; □, 120 μM ($[A]_{0.5}$ = 90 μM) at 24.5°. These concentrations of GABA are as close as could be achieved to the indicated concentrations, $[A]_{0.5}$, giving half-maximal response.

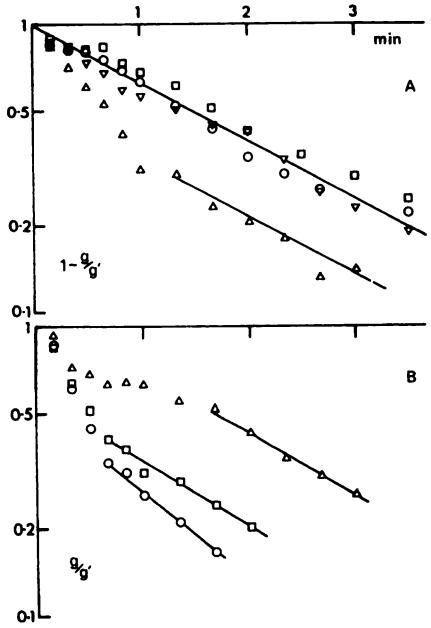

FIG. 10. *Kinetics of onset (A) and offset (B) of GABA action at 15°*

The method of plotting is explained in the legend to Fig. 7. The applications of GABA and washout were each of 10-min duration. The concentrations applied were: ○, 39 μM; □, 58 μM; ▽, 78 μM; △, 97 μM ($[A]_{0.5}$ = 52 μM). The rate constant of the slow exponential component of onset is 7.7×10^{-3} sec^{-1}. The rate constants of the slow components of offset are: ○, 1.2×10^{-2} sec^{-1}; □, 9.0×10^{-3} sec^{-1}; △, 8.3×10^{-3} sec^{-1}. All responses were recorded from the same fiber.

The concentration dependence of the kinetics at 15° is shown in Fig. 10. The onset of GABA action becomes obviously biphasic only when $[A]:[A]_{0.5}$ rises to about 2. The offsets are clearly slower than at higher temperatures (Fig. 7), and are biphasic at all concentrations. The offset of the highest GABA concentration is remarkable for the plateau between the two phases of the kinetics. This effect was exactly reproducible.

Membrane potential. The small changes in membrane polarization which accompany the conductance increase produced by GABA (Fig. 1) must be considered. They conceivably could exert an influence on membrane permeability, since large polarizations are known to do so in other preparations (13). Hyperpolarization in response to GABA was greater after replacement of chloride ions in the bathing solution with propionate (1). This suggests that the membrane is in fact poorly permeable to propionate ions and therefore can function as a propionate electrode (14). A slow depolarization in the continued presence of GABA follows the transient hyperpolarization (Fig. 1), possibly because of movement of chloride ions into the fiber, reducing the chloride Nernst potential. The responses sometimes became entirely depolarizing during the course of an experiment, particularly after repeated applications of high concentrations of GABA. However, at 15° the effect of GABA on membrane polarization either was not apparent or was too slow and too small to be identified with any confidence.

That the measured kinetics of conductance change was not influenced by these alterations in polarization is indicated by the following observations. (a) The voltage-current relationship of the membrane was linear over the range of polarization examined in these experiments. (b) The rate constant of the fastest response in Fig. 8 is 3.6×10^{-2} sec^{-1}, and that of the fastest response in Fig. 7A is 3.0×10^{-2} sec^{-1}. In the first example there was rapid hyperpolarization during onset, and in the second example depolarization alone was recorded. In general, the appearance of a fast phase of the kinetics was not found to be associated with particular changes in polarization. (c) GABA was also found not to affect the membrane potential when a "chloride saline" (1), in which all the sodium ions were replaced with choline, was used. Nevertheless, under these conditions, the rate constant of the onset of conductance change (at room temperature) agrees closely with the values reported here for the slow phase of onset.[4]

DISCUSSION

The slow exponential phase of the onset of GABA action resembles the kinetics of a first-order reaction. It cannot be the result of a macroscopic diffusion barrier, because the rate constant is independent of concentration. It cannot depend upon any process

[4] N. Brookes and S. Druckmann, unpublished observations.

utilizing metabolic energy, because it is relatively insensitive to temperature change. We are not able, therefore, to explain the slow phase of GABA action in terms of a model which relies on limited rate of access to the receptor (15) or active uptake of GABA from the extracellular space (16). The binding of GABA to the receptor, even if several orders of magnitude slower than substrate binding to enzymes (17), can be assumed to equilibrate very rapidly. Thus we are left with the possibility that we have observed here the rate of conformational transition of some structural element of the membrane, subsequent to the binding step.

Although there is an indication that similar arguments may apply to the fast component of GABA action, this is not clearly so at the present stage. The fast phase was not consistently observed to be exponential. We did not seek a limiting onset rate at high GABA concentration, but there was certainly no sign that such a limit existed even at substantially supramaximal concentrations. Thus any independence of concentration shown by the rate constant of the fast phase (Table 2) could only exist over a limited range. It is quite conceivable that the kinetics of the fast phase is in fact determined by diffusion limitation on the rate of access of GABA to the receptors. The rate of action of endogenously released transmitter could be very much faster, especially if transmitter were released in quantities sufficient to saturate the receptors. However, since the slow phase of GABA action does seem to be determined by the postsynaptic membrane, it is interesting to speculate that under physiological conditions concentrations may be always high enough to preclude the slow response.

Is the biphasic kinetics an expression of the existence of two distinct species of receptor with different affinities for GABA? If this is so, then the fast component is to be identified with the lower-affinity species, since its participation increases with concentration. Inspection of Table 2 shows that in this experiment the slow component must account for at least three-tenths of the maximum conductance change, and yet at high concentration the slow component of the kinetics has almost disappeared (Fig. 7). This inconsistency may be explained by the process of sensitization (Fig. 8), which seems to tell us that application of GABA promotes the conversion of the slow component to the fast. The objection which now arises is that during sensitization equilibrium responses to the same concentration show no consistent trend while the kinetics of their onset changes dramatically. It is difficult to accommodate this finding to the proposition that the fast and slow components are associated with different affinities for GABA.

It is emphasized that although our interpretation of sensitization rests mainly on the similarity of the components of the kinetics as they appear during (Fig. 8) and after (Fig. 7) sensitization, it is also true that some apparently more plausible explanations are inadmissible. If, for instance, saturation of GABA uptake or nonspecific binding were the cause of sensitization, the observed rate of response in an unsensitized preparation would be initially slow and then increasingly fast. This is the reverse of the experimental observation of a fast phase followed by a slow phase.

The plateau in the offset in Fig. 10B could be considered kinetic evidence of the "metastability" of responses observed in equilibrium studies (1). Attempts to account for slow offset rates in terms of leakage of intracellularly accumulated GABA meet the objection that, at least in crustacean muscle, GABA uptake has been found to be virtually unidirectional (16).

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